

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



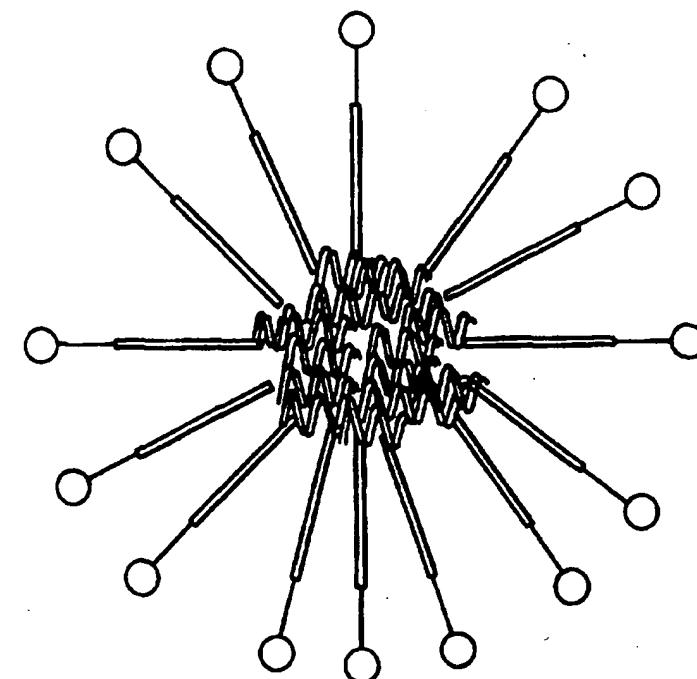
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>A61K 47/48, 48/00</b>		A1	(11) International Publication Number: <b>WO 96/15811</b>
			(43) International Publication Date: 30 May 1996 (30.05.96)
(21) International Application Number: <b>PCT/GB95/02706</b>		(81) Designated States: AL, AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG).	
(22) International Filing Date: 17 November 1995 (17.11.95)			
(30) Priority Data: 9423231.1 17 November 1994 (17.11.94) GB 9423308.7 18 November 1994 (18.11.94) GB 9512822.9 23 June 1995 (23.06.95) GB			
(71) Applicant (for all designated States except US): IMPERIAL COLLEGE OF SCIENCE, TECHNOLOGY & MEDICINE [GB/GB]; Sherfield Building, Imperial College, London SW7 2AZ (GB).		Published With international search report.	
(72) Inventors; and (75) Inventors/Applicants (for US only): HART, Stephen, Lewis [GB/GB]; Division of Cell and Molecular Biology, Institute of Child Health, 30 Guilford Street, London WC1N 1EH (GB). HARBOTTLE, Richard, Paul [GB/GB]; St. Mary's Hospital Medical School, Norfolk Place, London W2 1PG (GB).			
(74) Agents: CHAPMAN, Paul, William et al.; Kilburn & Strode, 30 John Street, London WC1N 2DD (GB).			

(54) Title: INTERNALISATION OF DNA, USING CONJUGATES OF POLY-L-LYSINE AND AN INTEGRIN RECEPTOR LIGAND

(57) Abstract

Compositions comprising DNA associated with a polycation moiety wherein the polycation moiety is itself coupled to an integrin receptor binding moiety are disclosed. Preferably, the integrin receptor binding moiety is a peptide, and the compositions can be used to deliver DNA to a cell where it will be expressed, for example, to treat a condition by gene therapy. In a preferred embodiment, the integrin receptor binding moiety comprises a peptide, in particular a cyclic peptide, comprising the sequence RGD. In a particularly preferred embodiment, the peptide comprises the sequence GGCRGDMFGC. Cyclic configuration in this sequence is imposed by virtue of the presence of two cysteine residues which can form a disulphide bond.



—○ CYCLIC RGD-CONTAINING PEPTIDE ATTACHED TO A POLYCATION

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Larvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

# INTERNALISATION OF DNA, USING CONJUGATES OF POLY-L-LYSINE AND AN INTEGRIN RECEPTOR LIGAND

The present invention relates to compositions comprising DNA associated with a polycation entity which is itself  
5 linked to an integrin specific binding moiety. The compositions of the invention can be used to deliver DNA to cells for internalisation and expression therein. In particular, therefore, the invention relates to methods of obtaining gene expression in cells to overcome genetic  
10 deficiencies.

In recent years, with the continuing identification of specific genes responsible for certain disease  
15 conditions, the concept of "gene therapy" has attracted a great deal of attention. The potential to deliver a new gene, or even part of a gene sequence, to a defective cell in order to correct such an inherent deficiency is an attractive one. There are, of course, inherent  
20 problems in such an approach. For instance, the DNA must be delivered in a form that will be taken up, or internalised, by the target cell. Furthermore, the DNA itself must be expressed effectively in the cell in order to overcome the genetic deficiency. Inherent in these  
25 problems is the additional one that the DNA itself, after having entered the cell, must be protected in some way to prevent its damage, or even destruction, by, for example, cellular enzymes.

One potential approach to this problem of  
30 internalisation, and protection, of DNA is disclosed in Hart et al (*J. Biol. Chem.*, 269, No 176: 12468-12474 (1994)). This approach exploits the presence of integrin receptors on cell surfaces for achieving internalisation of filamentous phage. Integrins are a super family of

heterodimeric cell adhesion molecules that consist of several different  $\alpha$  and  $\beta$  subunits. Their cellular function is to mediate the movement, shape and polarity of cells through binding with proteins of the extracellular matrix. In addition, integrins are exploited as receptors for cell entry by pathogenic bacteria, such as *Yersinia pseudotuberculosis* (Isberg, R., *Science*, 252: 934-938 (1991)) and *Bordetella pertussis* (Relamna et al, *Cell*, 61: 1375-1382 (1990)).

Hart et al (supra) found that displaying an integrin-binding peptide sequence on the surface of bacteriophage fd particles enabled the phage particles to be internalised by mammalian cells. However, no effective expression of DNA carried by phage particles was shown.

In addition, there are certain problems associated with the use of such particles to deliver DNA in this fashion. Firstly, there is a packaging size limitation governed by the size of the phage particle itself. Only genetic material up to a particular particle size could be delivered in this fashion. Secondly, the phage itself will only package single stranded genetic material, and this would not be effectively expressed in a mammalian cell system. Finally, the phage itself consists of other proteins and is a somewhat "messy" system for delivering DNA. It is possible that these additional components would have a material effect on whether or not genetic material was expressed.

Other approaches to delivering DNA into mammalian cells are disclosed in WO-A-9418834. Here, DNA was conjugated with a polyelectrolyte to form a complex which was then inserted into an embryonic cell, a germ cell or a germ

precursor cell. This method was disclosed primarily for producing transgenic animals. The methods disclosed in this document rely on either microinjection of the complex directly into the germ cell, or by having the polycation/DNA complex present in the culture medium and  
5 relying on uptake by the cells.

Cotton et al (PNAS USA, 87: 4033-4037 (1990)) used the natural iron-delivery protein transferrin, coupled to DNA  
10 binding polycations such as polylysine or protamine, to deliver DNA into human leukaemic cells. However, they also found that they required the use of other agents to effect the survival of the transfected DNA or to modulate transferrin receptor levels so as to increase the  
15 internalisation or uptake of the DNA itself. These steps included increasing the transferrin receptor density through treatment of the cells with the cell-permeable ion chelator, desferioxamine, interfering with the synthesis of heme with succinol acetone treatment or  
20 stimulating the degradation of heme with cobalt chloride treatment. In other words, effective uptake and expression of the DNA could only be achieved through the use of "co-factors" or co-treatments.

25 Thus, there exists a need for further and better methods of delivering DNA to a cell such that it will be internalised and expressed efficiently therein, preferably without the need for any other co-factors or co-treatments, and in a form which is not limited to  
30 genetic material of a particular size.

The approach taken by the present inventors is to use specific cell-surface integrin receptor binding moieties coupled to a polycation moiety which will bind to DNA.

The "DNA packages" will then bind to cell surface receptors and be internalised. It is also surprisingly been found that such an approach results in efficient expression of DNA so internalised, without the need for  
5 any co-factors or co-treatment. Nevertheless, co-factors can be used where desired. A preferred co-factor is chloroquine or any other factor which reduces endosomal degradative activity. The observation of improved expression in the presence of chloroquine may be because  
10 the peptide-DNA complex is internalised, at least in part, to endosomal compartments. Chloroquine is a weak buffer which is purported to prevent acidification of endosomal vesicles which limits the activity of endosomal degradative enzymes. Thus the internalised peptide-DNA  
15 complex has more opportunity to escape the endosome and avoid degradation. Other factors which might have a similar beneficial effect include ammonium chloride, another weak buffer which works like chloroquine; fusogenic peptides related to the N-terminus of the HA  
20 protein of influenza virus which mediate active membrane disruption and inactivated adenovirus capsids which also disrupt the membrane of the endosome.

One advantage of integrin receptor mediated  
25 internalisation is that large particles can be internalised, e.g. whole cells.

Thus, in a first aspect, the present invention provides a composition comprising DNA associated with a polycation  
30 wherein the polycation is coupled to an integrin receptor binding moiety.

In the present invention, "DNA" means single or double stranded DNA, either as complete coding sequences or

parts thereof and, in particular, refers to coding sequences for one or more genes.

5 "Integrin receptor binding moiety" means any moiety or species capable of specifically binding to integrin receptors found on the surface of cells. In particular, it refers to integrin receptor binding peptides capable of binding to integrin receptors.

10 "Association" of the DNA and the polycation occurs, for example, by virtue of charge-charge interaction, but other forms of association are equally applicable.

15 In a preferred embodiment, the integrin receptor binding moiety comprises a peptide, in particular a cyclic peptide, comprising the sequence RGD. In a particularly preferred embodiment, the peptide comprises the sequence GGCRGDMFGC. Cyclic configuration in this sequence is imposed by virtue of the presence of two cysteine  
20 residues which can form a disulphide bond.

The compositions of the present invention bind effectively to integrin receptors found on cell surfaces and are internalised. The DNA is then effectively  
25 expressed by the cell without the need for any other co-factors being present or the need for any co-treatment. Of course, co-factors or co-treatments can be used in conjunction with the present invention to boost expression levels even further.

30

The polycation moiety can be any suitable polycation capable of forming a complex with DNA. In particular, polycations such as polylysine can be used. The number of residues in the polycation can vary from a relatively

small number, up to quite long chains, or can be a mixture thereof. For example, polycations of from 3-1000, 3-500 or indeed 3-100 residues can be used. In particular, 10-16 cation residues are suitable, particularly 16. In one embodiment of the invention, therefore, the polycation consists of 10-16 polylysine residues, with 16 lysine residues being particularly preferred.

Without in any way being limited, it is believed that the polycation "tails" of the compositions of the invention associate with the DNA to be delivered, effectively forming a "package" with the integrin receptor binding moieties on the outside. The DNA composition can then bind to an integrin receptor on the cell surface and be internalised. The polycation may then act to protect the DNA from the cell's internal enzyme systems, enabling it to be integrated in the cell's genome and thus expressed.

In a further aspect, the present invention provides a DNA binding composition comprising an integrin receptor binding moiety coupled to a polycation. Preferably the integrin binding moiety is a peptide, as described above. Here coupling may occur to the C-terminus or to the N-terminus of the peptide. In one preferred embodiment, the polycation is polylysine.

This composition can then simply be brought into contact with DNA to "package" the DNA for delivery to a designated cell.

As discussed herein, compositions of the present invention enable the effective delivery of DNA to cells wherein it is internalised and expressed efficiently. In



this way, genetic deficiencies of particular cell types can be overcome by the delivery and expression of DNA sequences encoding correct or "native" proteins. One example where such an approach may be effective is in the treatment of cystic fibrosis. Thus, in other aspects, the present invention provides:

(a) the use of a composition of the invention in the manufacture of a medicament for the treatment or prophylaxis of a condition related to a genetic deficiency or modification;

(b) a composition of the invention for use in the treatment or prophylaxis of a condition related to a genetic deficiency or modification;

(c) a method for the treatment or prophylaxis of a condition related to a genetic deficiency comprising the step of administering to a subject a composition of the invention;

(d) a method for the transformation of a host cell comprising the step of bringing together the cell with a composition of the invention; such methods find use generally in transfection of cells, particularly mammalian cells;

(e) the use of a composition of the invention in the preparation of a medicament for the treatment or prophylaxis of a condition caused by a genetic deficiency or modification; and

(f) a pharmaceutical formulation comprising a composition of the invention together with one or

more pharmaceutically acceptable carriers, diluents or excipients.

5 Preferred features of each aspect of the invention are as for each other aspect *mutatis mutandis*.

The invention will now be described by reference to the following examples, which should not in anyway be construed as limiting the invention.

10

The examples refer to the figures in which:

15 FIGURE 1: shows a possible structure for the polycation-integrin receptor binding moiety/DNA complex.

20 FIGURE 2: shows levels of expression of a luciferase reporter protein in cells transformed with a composition of the invention, compared to suitable controls.

25 FIGURE 3: also shows levels of expression of a luciferase reporter protein in cells with a composition of the present invention, compared to suitable controls, but here the composition is different from that used in respect of Figure 2.

30 FIGURE 4: shows levels of expression of a luciferase reporter protein in cells transformed with various compositions of the invention;

FIGURE 5: shows the effect of chloroquine on expression of a luciferase reporter protein in COS-7 cells;

FIGURE 6: shows the effect of chloroquine on expression of a luciferase reporter protein in endothelial cells;

5       FIGURE 7: shows the results of expression of a luciferase reporter protein in COS-7 cells using differing ratios of RGD peptide:DNA, in the presence and absence of chloroquine;

10       FIGURE 8: shows the results of expression of a luciferase reporter protein in endothelial cells using differing ratios of RGD peptide:DNA, in the presence and absence of chloroquine; and

15       FIGURE 9: shows the results of comparing transfection of endothelial cells with varying ratios of K10 peptide:DNA and of K16 peptide:DNA.

20       EXAMPLE 1: Preparation of a first Polylysine-Integrin receptor binding peptide.

The peptide sequence GGCRGDMFGC(K)<sub>16</sub> was synthesised as follows:

25

(a) the peptide was synthesised on ABI model 431A solid-phase batch peptide synthesiser using Wang HMP resin and Fmoc-cleavage strategy;

30

(b) the linear peptide was cleaved from the resin using 5 ml of a scavenger mixture (0.75 g phenol, 0.25 ml EDT, 0.5 ml thioanisole, 0.5 ml deionised H<sub>2</sub>O, 10 ml TFA), the mixture was stirred for 2 hrs at room temperature and was then filtered over sinter

into ice-cold MTBE. This was then stored at  $-18^{\circ}\text{C}$  before being spun down, washed with 3 x 6 ml MTBE before being dried in vacuo and redissolved in  $\text{H}_2\text{O}$  and freeze dried;

5

(c) cyclisation of the peptide was carried out in 5%  $\text{AcOH}/20\%$  DMSO v/v, buffered to pH 6 by 0.88  $\text{NH}_3(\text{aq})$ , with stirring for 24 hrs at room temperature. Finally, it was diluted (x 3) using deionised water;

10

(d) purification by ion-exchange chromatography was carried out using Mono-S resin, 50 mM HEPES buffer (pH 7.6) on Pharmacia FPLC system (monitored at 280 nm/UV-Hg lamp);

15

(e) fractions were assayed for effects on cell cultures;

20

(f) positive fractions were desalted using P2 Biogel and 0.1% aq TFA; and

25

(g) further desalting was carried out using reverse-phase chromatography and 0.1% aq TFA on an FPLC system (monitored at 214 nm/Zn lamp).

30

**EXAMPLE 2:** Internalisation and expression of a reporter gene using the polylysine-integrin receptor binding peptide described in Example 1.

5  $\mu\text{g}$  of a luciferase reporter gene plasmid (pGL2 promega) was complexed with either the RGD-polylysine construct

(possible structure of complex is shown in Figure 1) or an equal concentration of polylysine in 100  $\mu$ l of OptiMem media (Gibco). The DNA/peptide complexes and also a DNA only control were then applied to 50% confluent cultured (Caco-2) colonic epithelial cells which were then allowed to express for 48 hours. The cells were then harvested and the cellular protein analysed for luciferase activity (Relative Light Units). The activity shown in Figure 2 is adjusted to represent activity from 1 mg of cellular protein. In Figure 2 the term "RGD peptide" is used to indicate the polylysine-integrin receptor binding protein described in Example 1.

**EXAMPLE 3:** Preparation of a second polylysine-integrin receptor binding peptide.

The peptide sequence (K)<sub>16</sub>GGCRGDMFGCA was synthesised. This can be done using analogous techniques to those used in respect of Example 1. This peptide has a similar sequence to that referred to in Example 1, the main difference being that the polylysine region was present at the N-terminus rather than the C-terminus.

**EXAMPLE 4:** Internalisation and expression of reporter gene using the polylysine-integrin receptor binding peptide described in Example 3

The procedure described in Example 2 was repeated but using the polylysine-integrin receptor binding peptide described in Example 3 instead of the polylysine-integrin receptor binding peptide described in Example 1. Control experiments were also performed. The results are shown in Figure 3, wherein:

- DNA control = 5  $\mu$ g of PGL2 plasmid DNA  
PolyK = poly-L-lysine  
RGD = newly synthesised RGD-containing peptide linked to polylysine (described in Example 3)  
5 Chlor = 100  $\mu$ M chloroquine  
2 x RGD+DNA = twice as much as peptide, DNA same (5  $\mu$ g)  
10 5 x RGD+DNA = five times as much peptide.

An indirect comparison of optimal peptide-DNA transfection efficiencies in the absence of chloroquine suggests that the peptide prepared in Example 3 ( $\pm 6 \times 10^7$  RLU/mg) is more efficient than the peptide prepared in Example 1 ( $\pm 3.5 \times 10^5$  RLU/mg in Figure 2) at delivery and expression of the luciferase reporter gene. The addition of chloroquine improved expression a further 2 fold approximately, suggesting that endosomal degradation is limiting the expression levels somewhat. It is interesting in comparison, however, that, in some circumstances, the efficiency of the transferrin-polylysine receptor-mediated gene delivery system was improved by more than 1,000-fold in the presence of chloroquine. The high level of expression by the RGD-polylysine peptide without co-factors and the relatively small improvement in enhancement with chloroquine is surprising.

30 **EXAMPLE 5:** Transfection of Caco-2 cells with K16 RGD peptide and effect of chloroquine.

The polylysine-integrin receptor binding peptide of Example 1 was used in a repeat of the procedure of

Example 2, but using 1  $\mu$ g of luciferase reporter gene plasmid. The effect of chloroquine was also investigated. The results are shown in Figure 4. Highest transfection levels were achieved with 2 x RGD peptide and 100 nm chloroquine, these transfection levels being approximately 10-fold lower than those achieved with lipofectamine. It can again be seen that chloroquine gave a relatively small improvement with 2 x RGD peptide + DNA, although with RGD-peptide + DNA the improvement was greater.

**EXAMPLE 6:** Effects of chloroquine on transfection of COS-7 cells with RGD peptides.

The polylysine-integrin receptor binding peptide of Example 1 was again used. Essentially the methodology of Example 2 was followed with the substitution of COS-7 cells, and the use of different concentrations of chloroquine. The results are shown in Figure 5. It can be seen that 200  $\mu$ m chloroquine gave a 4-fold increase of expression, compared with no chloroquine. However, at these levels, cytopathic effects of chloroquine were apparent.

**EXAMPLE 7:** Effects of chloroquine on transfection of endothelial cells with RGD peptides.

This was a repeat of Example 6 using ECV 304 endothelial cells. The results are shown in Figure 6. For these cells, the highest level of expression was obtained in the absence of chloroquine. Although increasing levels of chloroquine restored expression to some extent, complete restoration was not achieved.

**EXAMPLE 8:** Transfection of COS-7 cells with RGD-polylysine peptides.

5 The methodology of Example 2 was repeated using COS-7 cells. In this experiment, different ratios of the K16 RGD peptide DNA were used with and without 100  $\mu$ m chloroquine, with the results shown in Figure 7. It can be seen that the optimum ratio was 37.5:1 in both the absence and presence of chloroquine.

10

**EXAMPLE 9:** Transfection of endothelial cells with RGD-polylysine peptides.

15 This was a repeat of Example 8 using ECV 304 endothelial cells. The results are shown in Figure 8.

**EXAMPLE 10:** Transfection of endothelial cells comparing K10 and K16 RGD peptides with lipofectamine.

20

ECV 304 endothelial cells were transfected with the optimised ratio of K16 RGD peptide-DNA complex (37.5:1) derived from Example 9. In addition, a range of ratios of K10 RGD peptide-DNA complex were also investigated, as well as transfection using lipofectamine. The results are shown in Figure 9, with the optimised K16 RGD-DNA complex beingz the most efficient.

25



CLAIMS

1. A composition comprising DNA associated with a polycation moiety wherein the polycation moiety is itself coupled to an integrin receptor binding moiety.  
5
2. A composition as claimed in claim 1 wherein the integrin receptor binding moiety is a peptide.
- 10 3. A composition as claimed in claim 2 wherein the peptide comprises the sequence RGD.
4. A composition as claimed in claim 2 or claim 3 wherein the peptide is cyclic.  
15
5. A composition as claimed in claim 4 wherein the peptide comprises at least two cysteine residues capable of forming a disulphide bond.
- 20 6. A composition as claimed in claim 5 wherein the peptide comprises the sequence GGCRGDMFGC.
7. A composition as claimed in any one of claims 1 to 6 wherein the polycation moiety consists of polylysine.  
25
8. A composition as claimed in any one of claims 1 to 7 wherein the polycation moiety consists of 10-16 cation residues.
- 30 9. A composition as claimed in claim 8 wherein the polycation moiety consists of 16 cation residues.
10. A composition as claimed in claim 9 wherein the ratio of polycation moiety to DNA is 37.5:1.

11. A composition as claimed in any one of claims 1 to 8 wherein the DNA is double stranded.
12. A composition as claimed in claim 11 wherein the DNA  
5 comprises one or more complete gene coding sequences.
13. A composition as claimed in claim 12 wherein the DNA includes the coding sequence of the cystic fibrosis gene.
- 10 14. A DNA binding composition comprising a polycation moiety coupled to an integrin receptor binding moiety.
- 15 15. A composition as claimed in claim 14 modified by any one or more of the features of claims 2 to 9.
- 16 16. A method of preparing a composition as defined in any one of claims 1 to 10 which comprises the step of bringing together a composition as defined in claim 14 or claim 15 with DNA.
- 20 17. A method as claimed in claim 16 wherein the DNA is as defined in any one of claims 11 to 13.
- 25 18. The use of a composition as defined in claim 14 or claim 15 in the preparation of a composition as defined in any one of claims 1 to 13.
- 30 19. A method for expressing DNA in a host cell which comprises the step of bringing together the host cell and a composition as defined in any one of claims 1 to 13.
20. A method as claimed in claim 19 wherein the host cell is a mammalian cell.

21. The use of a composition as defined in any one of claims 1 to 15 in the preparation of a medicament for the treatment or prophylaxis of a condition caused by a genetic deficiency or modification.

5

22. A pharmaceutical formulation comprising a composition as defined in any one of claims 1 to 13 together with one or more pharmaceutically acceptable carrier, diluent and/or excipient.

10

23. A composition as defined in any one of claims 1 to 15 for use in medicine.

15

24. A method for the treatment or prophylaxis of a condition caused by a genetic deficiency or modification comprising administering to a subject a composition as defined in any one of claims 1 to 13 or a pharmaceutical formulation as defined in claim 22.

20

25. A method for preparing a composition as defined in claim 14 or claim 15 comprising the steps of:

(a) synthesising an integrin receptor binding peptide coupled to a polylysine moiety;

25

(b) subjecting the peptide to a cyclisation step;

(c) purifying the mixture of peptides obtained by ion-exchange chromatography;

30

(d) assaying fractions for presence of integrin receptor binding peptide;

(e) desalting fractions containing integrin

receptor binding peptide; and

5 (f) further desalting the integrin receptor binding peptide containing fractions using reverse phase chromatography.

26. A composition according to any of claims 1 to 13, further comprising a factor which reduces endosomal degradative activity.

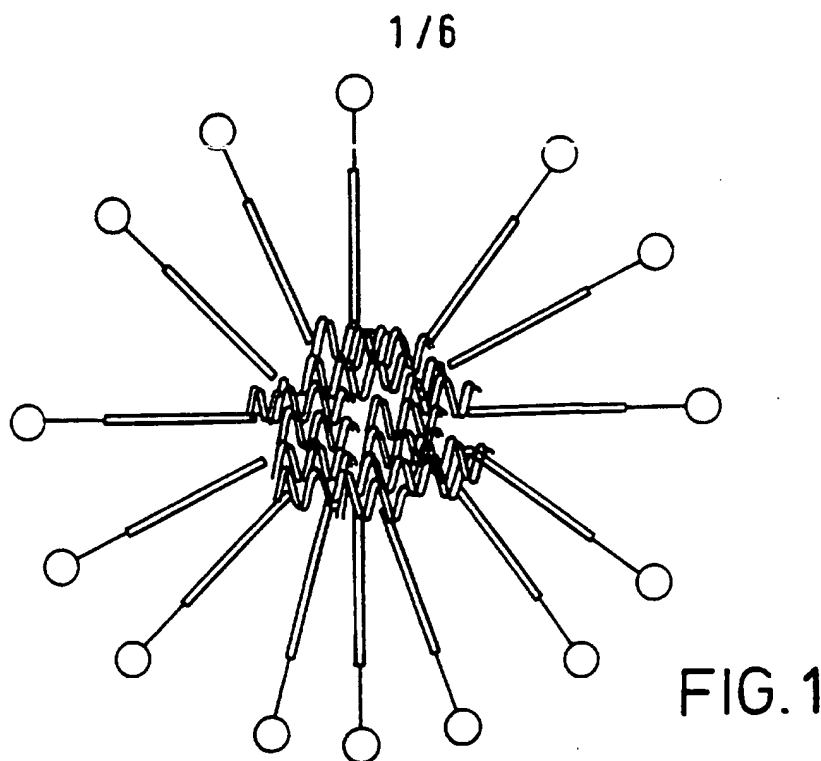

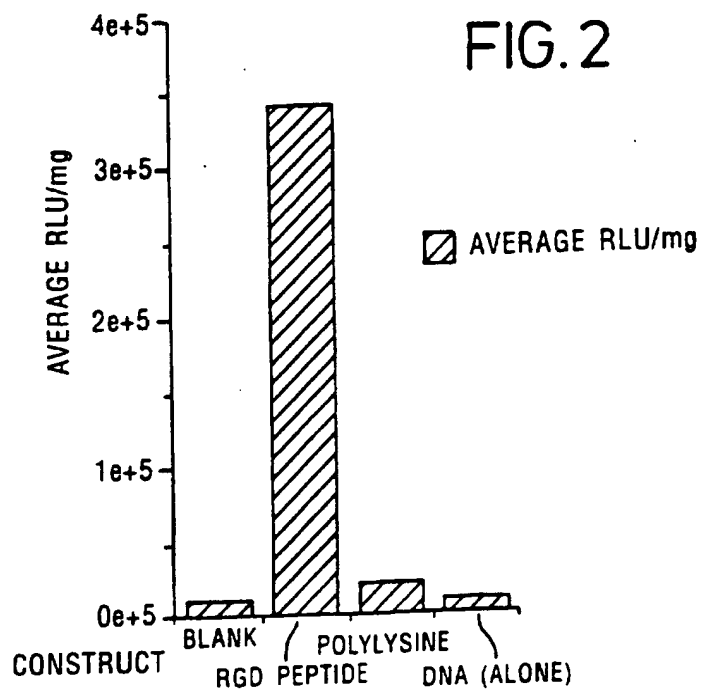
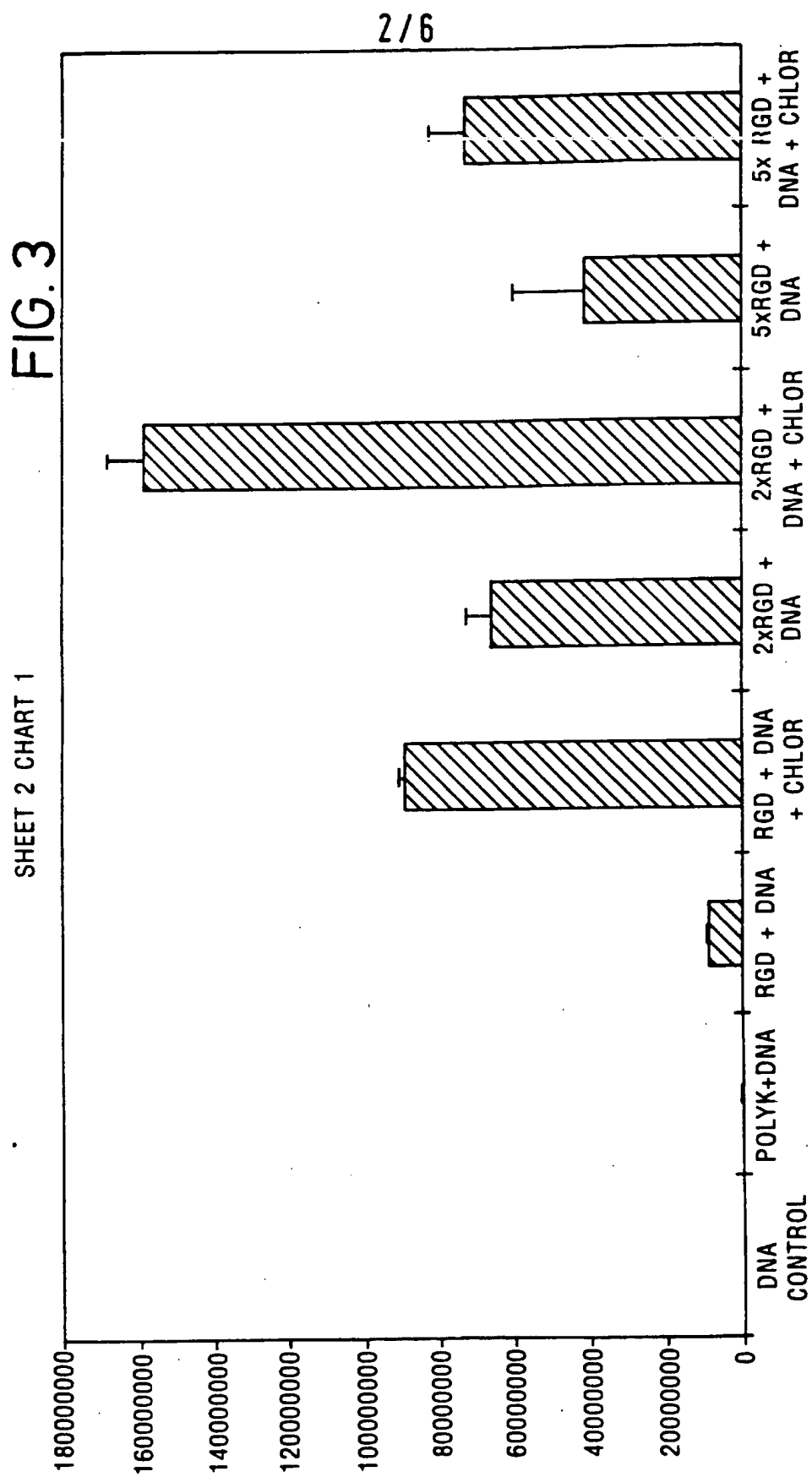


FIG.1

 CYCLIC RGD-CONTAINING PEPTIDE  
ATTACHED TO A POLYCATION





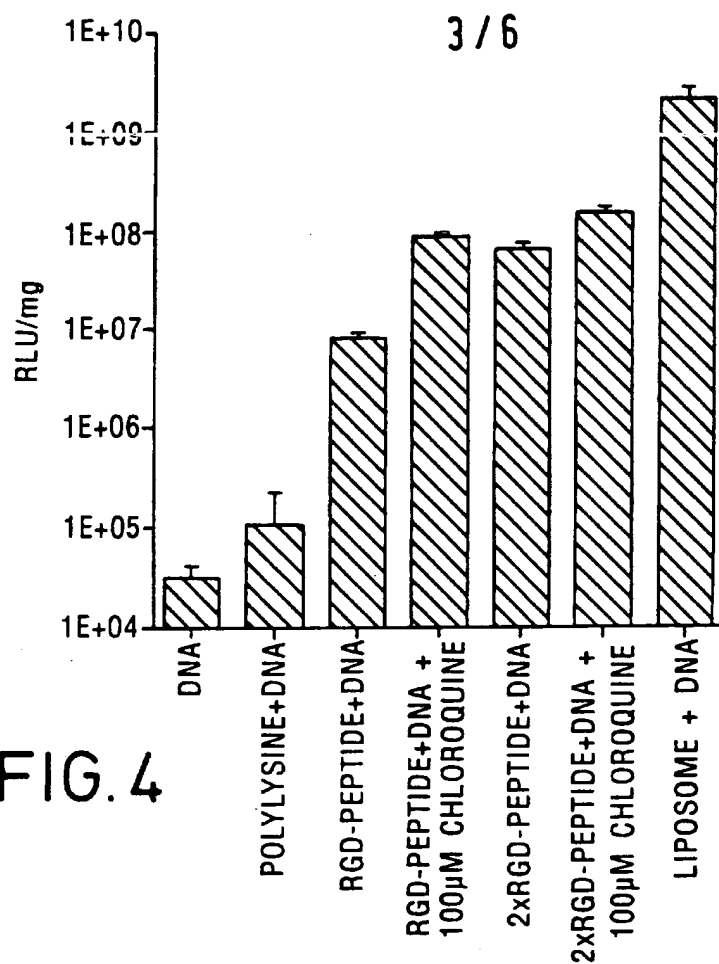


FIG. 4

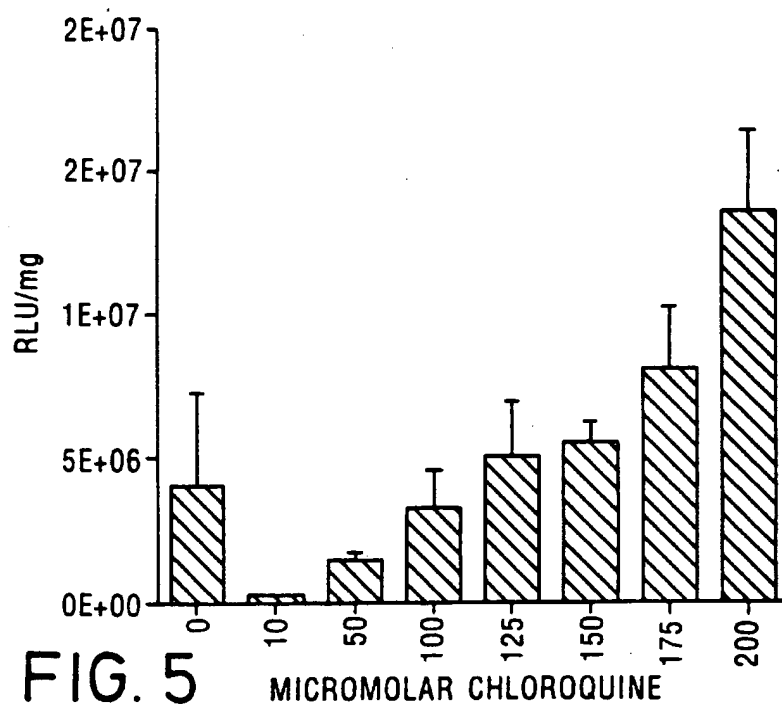
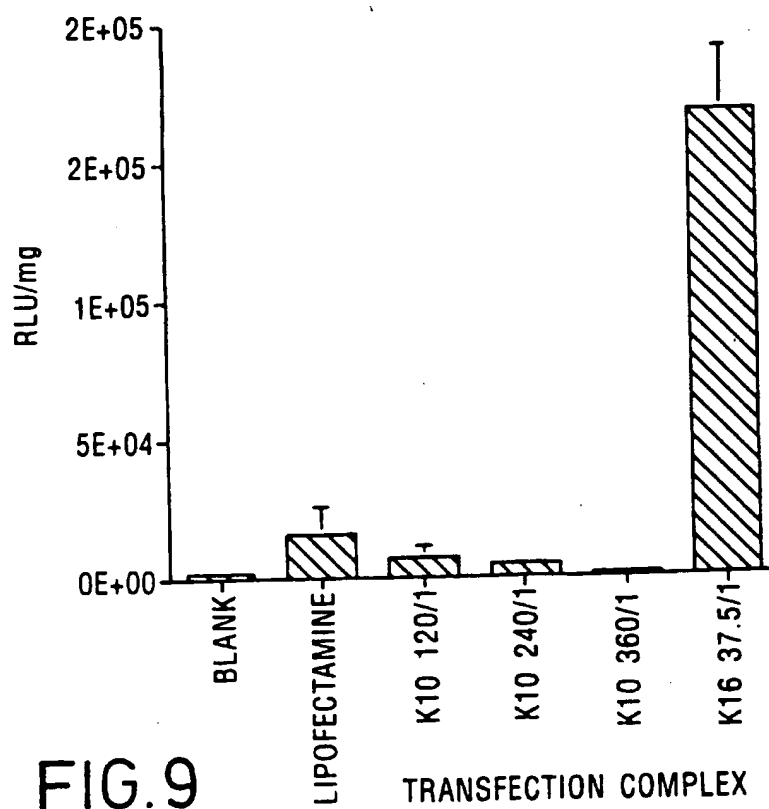
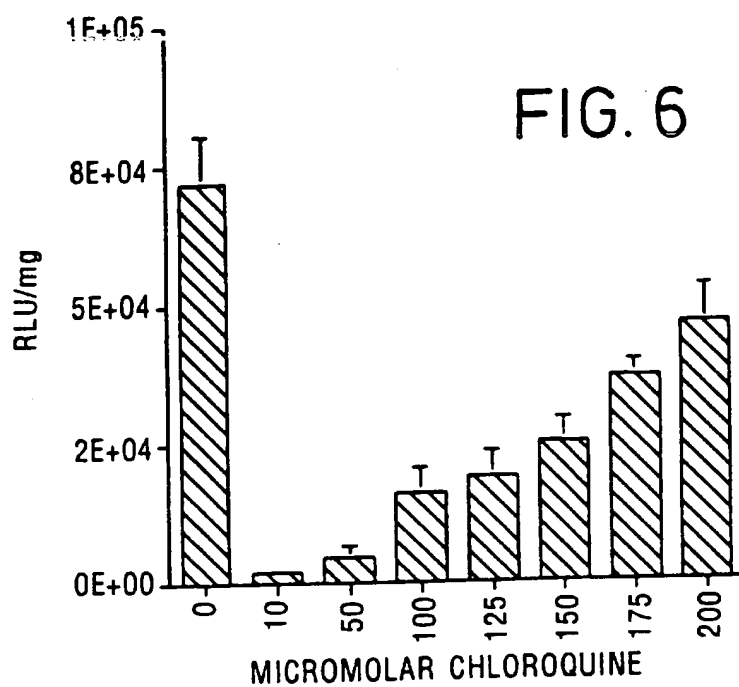


FIG. 5

4/6





5 / 6

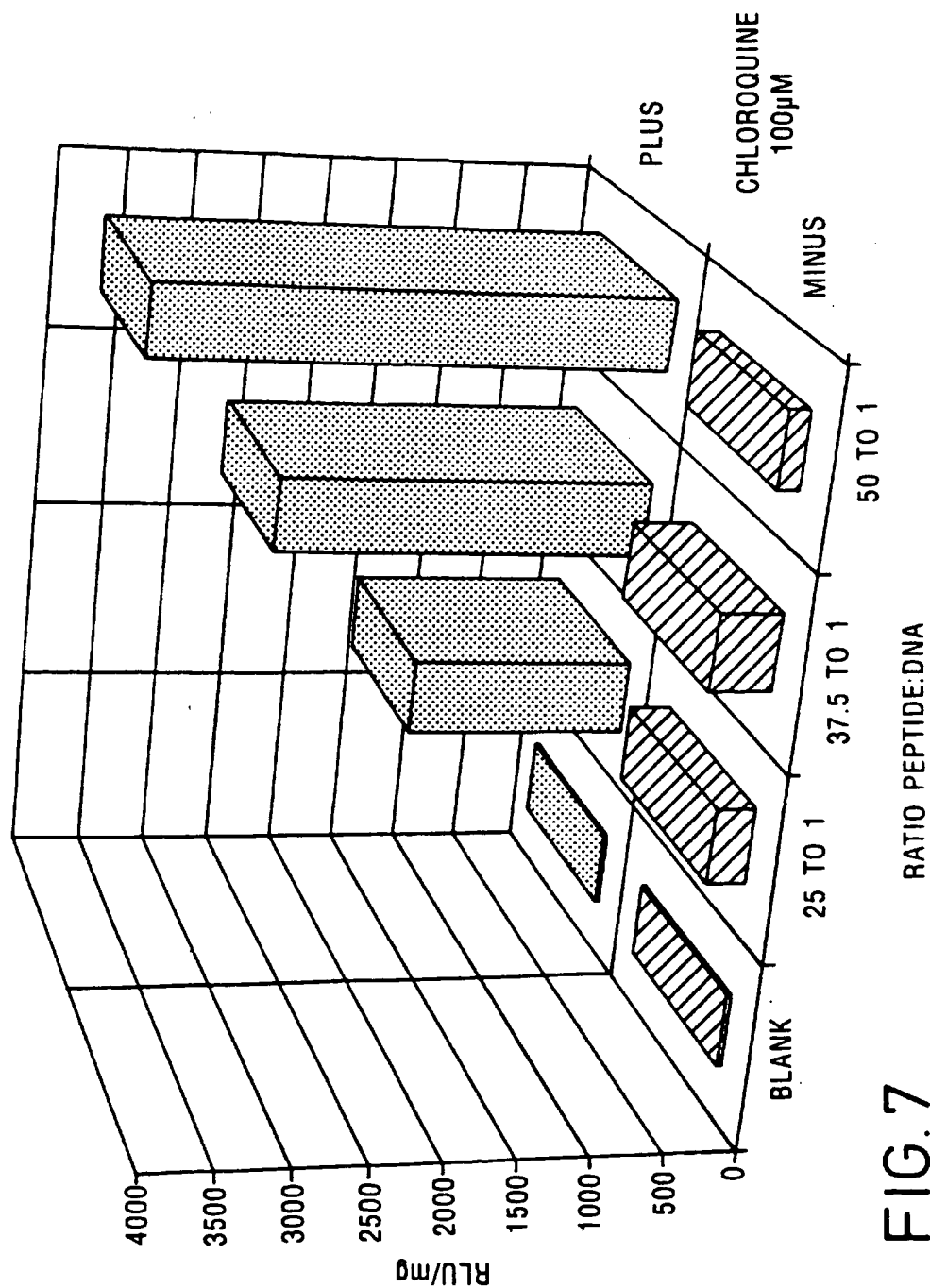


FIG. 7

6 / 6

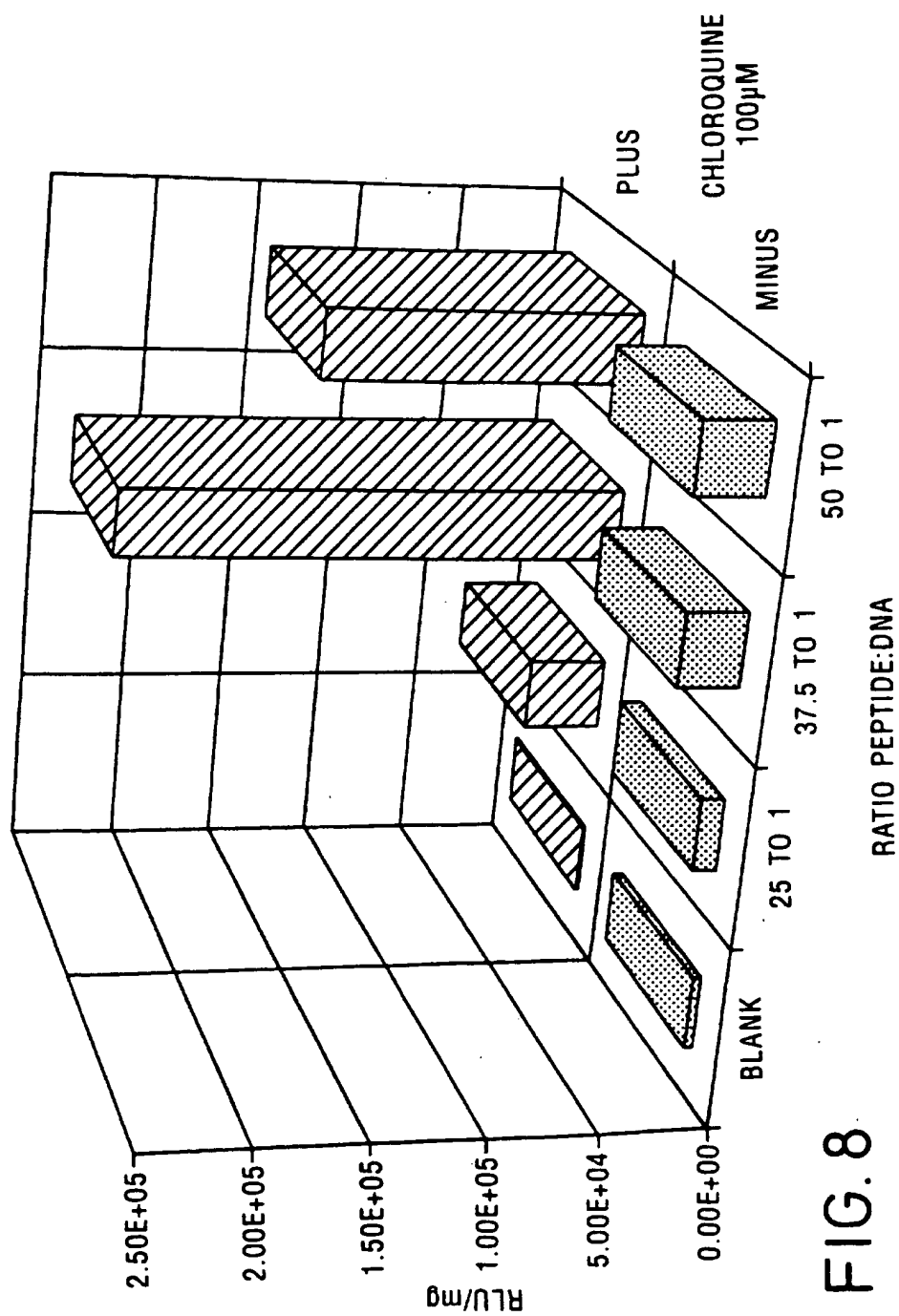


FIG. 8

# INTERNATIONAL SEARCH REPORT

International Application No  
PC1/GB 95/02706

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 A61K47/48 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	COTTEN M. ET AL 'Receptor-mediated transport of DNA into eukaryotic cells', METHODS ENZYMOL., 1993, VOL. 217, PART H, PAGE(S) 618-644, USA XP 000562730 see page 619 see figure 1 see page 622 - page 623 ---	1-26
X	J. BIOL. CHEM., 29-4-1994, VOL. 269, NO. 17, PAGE(S) 12468-1247, USA, HART S.L. ET AL 'Cell binding and internalization by filamentous phage displaying a cyclic Arg-Gly-Asp-containing peptide' cited in the application see abstract see page 12473, right column ---	1-26
-/--		

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- \*A\* document member of the same patent family

Date of the actual completion of the international search

20 February 1996

Date of mailing of the international search report

05.03.96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax (+31-70) 340-3016

Authorized officer

Dullaart, A

# INTERNATIONAL SEARCH REPORT

International Application No

PC/GB 95/02706

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EMBO J., 1993, VOL. 12, NO. 5, PAGE(S) 1887-1895, OXFORD, GB, VAN NHIEU G.T. ET AL 'Bacterial internalization mediated by betal chain integrins is determined by ligand affinity and receptor density' see abstract see page 1890 - page 1892 ---	1-26
Y	CHEMICAL ABSTRACTS, vol. 122, no. 21, 22 May 1995 Columbus, Ohio, US; abstract no. 262311, ISBERG, RALPH R. 'Internalization of microbial pathogens by integrin receptors and the binding of the Yersinia pseudotuberculosis invasin protein' see abstract & INTEGRINS BIOL. PROBL. 1994, PAGES 197-216. EDITOR(S): TAKADA, YOSHIKAZU., 1994 BOCA RATON, US, ---	1-26
Y	DATABASE WPI Section Ch, Week 9238 Derwent Publications Ltd., London, GB; Class B04, AN 92-313681 & JP,A,04 221 397 ( FUJI PHOTO FILM CO LTD) , 11 August 1992 see abstract & CHEMICAL ABSTRACTS, vol. 118, no. 21, 24 May 1993 Columbus, Ohio, US; abstract no. 213547, page 972; column 1; see abstract & PATENT ABSTRACTS OF JAPAN vol. 16 no. 566 (C-1009) ,8 December 1992 see abstract ---	1-26
Y	CELL, 1993, VOL. 73, NO. 2, PAGES 309-19, 1993 CAMBRIDGE, US, WICKHAM, THOMAS J. ET AL 'Integrins.alpha.v.beta.3 and.alpha.v.beta.5 promote adenovirus internalization but not virus attachment' see abstract see figures --- -/--	1-26

# INTERNATIONAL SEARCH REPORT

International Application No  
PL 1/GB 95/02706

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. BIOL. CHEM., VOL. 269, NO. 32, PAGES 20233-8, 12 August 1994 PFAFF, MARTIN ET AL 'Selective recognition of cyclic RGD peptides of NMR defined conformation by.alpha.IIb.beta.3,.alpha.V.beta.3, and.alpha.5.beta.1 integrins' see abstract see paragraph RESULTS see figures see tables 1,2 see paragraph DISCUSSION ---	1-26
Y	TRENDS IN MICROBIOLOGY, 1994, VOL. 2, NO. 1, PAGE(S) 10-14., XP 000562727 ISBERG R R ET AL 'Binding and internalization of microorganisms by integrin receptors' see abstract see paragraph Perspective ---	1-26
Y	WO,A,93 23085 (DIATECH, INC.) 25 November 1993 see abstract see examples see figures see claims ---	1-26
Y	NAT. IMMUN., VOL. 13, NO. 2-3, PAGE(S) 141-164, March 1994 - June 1994 , BASEL, CH, XP 000562731 CURIEL D.T. 'High-efficiency gene transfer employing Adenovirus-polylysine-DNA complexes' see page 144 see page 145 see figures 1,3-8,10 ---	1-26
Y	EXP. CELL RES., 1992, VOL. 199, NO. 2, PAGE(S) 323-329, XP 000562696 ROSENKRANZ A.A. ET AL 'Receptor-mediated endocytosis and nuclear transport of a transfecting DNA construct' see abstract see paragraph Materials and methods see paragraph Results --- -/--	1-26

## INTERNATIONAL SEARCH REPORT

Int'l Application No

PCT/GB 95/02706

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF BIOLOGICAL CHEMISTRY, 1993, VOL. 268, NO. 15, PAGE(S) 11265-11271., CHOWDHURY N R ET AL 'FATE OF DNA TARGETED TO THE LIVER BY ASIALOGLYCOPROTEIN RECEPTOR-MEDIATED ENDOCYTOSIS IN-VIVO PROLONGED PERSISTENCE IN CYTOPLASMIC VESICLES AFTER PARTIAL HEPATECTOMY' see page 11265 - page 11266 see page 11270 see page 11271 ---	1-26
Y	J BIOL CHEM, APR 5 1987, VOL. 262, NO. 10, PAGE(S) 4429-32, WU GY ET AL 'Receptor-mediated in vitro gene transformation by a soluble DNA carrier system' see abstract see paragraph Experimental procedures see figure 2 see paragraph Results and discussion ---	1-26
L	& J BIOL CHEM, 1988 JAN 5, VOL. 263, NO. 1, PAGE 588, 'published erratum' L: provided for complete information ---	
Y	BIOCHEMISTRY, FEB 9 1988, VOL. 27, NO. 3, PAGE(S) 887-92, WU GY ET AL 'Evidence for targeted gene delivery to Hep G2 hepatoma cells in vitro.' see abstract see paragraph Materials and methods see figures 4,5 see tables 2,3 see paragraph RESULTS ---	1-26
Y	PROC. NATL ACAD. SCI. U. S. A., 1991, VOL. 88, NO. 19, PAGE(S) 8850-8854, US, CURIEL D.T. ET AL 'Adenovirus enhancement of transferrin-polylysine-mediated gene delivery' see abstract see paragraph Materials and methods see figures ---	1-26
Y	NEUROLOGY, APRIL 1994, VOL. 44, NO. 4 SUPPL. 2, PAGE(S) A268, ABSTRACT NO. 567S, XP 000562694 ASCADI G ET AL 'Optimization of in vivo gene transfer into skeletal muscle cells by adenovirus vector' see abstract & 46TH ANNUAL MEETING OF THE AMERICAN ACADEMY OF NEUROLOGY, WASHINGTON, D.C., USA, MAY 1-7, 1994. ---	1-26
	---	

-/--

# INTERNATIONAL SEARCH REPORT

Int ional Application No  
PCT/GB 95/02706

C.(Continuation) DOCUMENTS C NSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ANN. NEW YORK ACAD. SCI., 1994, VOL. 716, PAGE(S) 36-58, US, XP 000562729 CURIEL D.T. ET AL 'High-efficiency gene transfer mediated by adenovirus-polylysine- DNA complexes' see figures	1-26
P,X	--- KEYSTONE SYMPOSIUM ON GENE THERAPY AND MOLECULAR MEDICINE, STEAMBOAT SPRINGS, COLORADO, US, MARCH 26-APRIL 1, 1995. PUBLISHED IN: JOURNAL OF CELLULAR BIOCHEMISTRY, SUPPLEMENT 21A, 1995, PAGE 394, ABSTRACT NO. C6-321, HARBOTTLE R ET AL 'RGD -mediated gene delivery and expression in epithelial cells.' see abstract -----	1-26

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PC/GB 95/02706

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9323085	25-11-93	AU-B- 4384593	13-12-93
		CA-A- 2136330	25-11-93
		EP-A- 0641222	08-03-95
		JP-T- 7508289	14-09-95
-----			